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Human Herpesvirus 6 Infection in Autologous Bone Marrow Transplant Recipients: A Prospective Study

Donatella Moschettini,[1]* Piero Galieni,[2] Pier Egisto Valensin,[1] Daniele Laszlo,[2] Giulia Scalia,[2] Monica Tozzi,[2] Francesco Lauria,[2] and Donatella Donati[1]

[1]*Dipartimento di Biologia Molecolare, Sezione di Microbiologia, Università di Siena, Siena, Italy*
[2]*Dipartimento di Ematologia, Università di Siena, Siena, Italy*

After primary infection in early life, human herpesvirus 6 (HHV-6) remains latent in the body and may reactivate in subjects with poor immune status. A 180-day longitudinal study of HHV-6 infection was carried out in 23 autologous bone marrow transplant recipients to evaluate reactivation of HHV-6; two of these patients underwent a double transplant. The patients were monitored prospectively for HHV-6 DNA in peripheral blood mononuclear cells (PBMC) by hot start nested PCR. Positive samples were typed by the enzymatic restriction protocol. Positive plasma samples were also tested for HHV-6 DNA. Antibodies against HHV-6 were measured by immunofluorescence. Five and two out of 23 patients had intermittent and persistent positivity to HHV-6 DNA in PBMCs, respectively; four patients carried variant B, and the other three patients both A and B. None of the respective plasma samples were positive. Two patients were positive for HHV-6 antibodies. Since the significance of HHV-6 DNA in PBMCs is unclear, these findings do not necessarily indicate active infection but may be due to mild immunosuppression in autologous BMT recipients. ***J. Med. Virol. 60:39–42, 2000.***

KEY WORDS: HHV-6; polymerase chain reaction (PCR); reactivation; autologous BMT

INTRODUCTION

Human herpesvirus type 6 (HHV-6) is a β-herpesvirus [Braun et al., 1997] first isolated from peripheral blood mononuclear cells (PBMC) [Salahuddin et al., 1986], is the causative agent of exanthem subitum [Yamanishi et al., 1988]. After primary infection in early life the virus remains latent in the body [Luppi et al., 1993], and may reactivate in subjects with poor immune status. The pathogenic role of HHV-6 has been documented most frequently in allogeneic bone marrow transplant (BMT) recipients. In these immunodepressed patients, HHV-6 infection can cause severe interstitial pneumonitis [Carrigan et al., 1991; Cone et al., 1993], suppresses bone marrow function [Drobyski et al., 1993; Carrigan and Knox, 1994] and has been associated with graft versus host disease (GVHD) [Wilborn et al., 1994]. HHV-6 may also be responsible for fatal encephalitis/meningitis in BMT recipients [Drobyski et al, 1994; Rieux et al, 1998].

Since the immune status of autologous BMT recipients is only influenced relatively by conditioning therapy, the occurrence of active HHV-6 infection in a population of autologous BMT recipients monitored for 180 days after the transplant was evaluated. HHV-6 DNA in PBMCs and plasma samples, HHV-6 typing and serology were the parameters taken into account. The results were compared with those of a healthy control group.

MATERIALS AND METHODS

Patients

Twenty-three patients (mean age 46.8 years; 12 females and 11 males) undergoing autologous BMT at the Department of Hematology of Siena University were monitored for HHV-6 DNA in PBMCs and plasma specimens and for serum antibodies. Two patients underwent double autologous BMT. Ten healthy subjects were used as negative control. Diagnosis before transplant was non-Hodgkin lymphoma in 16 patients (two received double autologous BMT), Hodgkin lymphoma in one patient, acute myeloblastic leukemia in two patients, multiple myeloma in one patient and breast cancer in three patients. The conditioning regimen was BAVC (BCNU, Ara-C, VP-16, Cyclophosphamide) or BEAM (BCNU, VP-16, Ara-C, Melphalan) for non-Hodgkin lymphoma and Hodgkin disease patients, BU/MELP (busulphane, melphalan) for acute myeloblastic

*Correspondence to : Donatella Moschettini, Dipartimento di Biologia Molecolare, Sezione di Microbiologia, Università di Siena, Via Laterina, 8, 53100 Siena, Italy.
E-mail : Moschettini@unisi.it

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leukemia, MELP for multiple myeloma and TT/MELP (Thio Tepa, Melphalan) for breast cancer.

Sampling

Ten ml of citrated blood were obtained from the patients 0, 7, 14, 30, 45, 60, 90 and 180 days after the transplant. PBMCs were separated by Ficoll (Pharmacia, Uppsala, Sweden) gradient and stored as dry pellets at −20°C; plasma samples were divided into 2 aliquots which were stored at −20°C after previous decomplementation and −80°C for serology and DNA detection, respectively. Ten ml of citrated blood from healthy subjects was obtained 0, 3 and 6 months after recruitment and processed as above.

Hot Start Polymerase Chain Reaction (PCR)

Extraction of DNA from PBMC was carried out by a salting out method [Miller et al., 1988]. A QIAamp blood kit (Qiagen, Germany) was used according the manufacturer's instructions to extract purified DNA from plasma samples. To verify the reliability of this method, the silica particle procedure was also applied to plasma samples [Boom et al., 1990]. The recommendations of Kwok and Higuchi [1989] were followed to avoid contamination of the PCR samples. The suitability of each sample for PCR testing was assessed by amplifying a human beta-globin gene region. The HSB-2 cell line was infected with HHV-6 (GS strain) and viral DNA was extracted to obtain a positive control for the PCR experiments.

One microgram of cellular DNA and 10 μl of plasma were used separately as template for the first amplification reaction, performed in 50 μl of reaction mixture containing 50 mM KCl, 10 mM Tris-HCl, pH 8.6, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 75 μM each of the four deoxynucleotide triphosphates (dNTPs), 10 pmol primer pair H6 1/H6 2 [Moschettini et al., 1996], and 0.7 U *Taq* DNA polymerase. After the first denaturing step (94°C for 5 min), 30 cycles of the first round PCR were carried out as follows: 54°C for 40 sec, 72°C for 30 sec, 94°C for 40 sec, with a final extension at 72°C for 2 min.

The second round PCR was carried out by adding 1.5 μl of the first PCR product to 50 μl of the same PCR reaction mixture, except that 100 μM dNTPs, 10 pmol primer pairs E/A and 0.3 U *Taq* DNA polymerase were used. Thirty cycles of the inner PCR were performed as follows: 56°C for 40 sec, 72°C for 25 sec, 94°C for 40 sec, with the same final extension of the outer PCR. Ten microliters of the final PCR product was electrophoresed on agarose gel (2.4% NuSieve, 0.6% Seakem; FMC, USA) and visualised by ethidium bromide staining [Moschettini et al., 1998]. Our PCR detected as few as 5 copies of the viral target in 1 μg of background DNA.

HHV-6 variants were characterised by enzymatic restriction of PCR products according to the method by Secchiero et al. [1995].

Serology

HSB-2 cells were infected with HHV-6 (GS strain, kindly donated by Prof. Ceccherini-Nelli, Pisa, Italy); after 48–72 hours, the presence of CPE was evaluated and the infected cells were harvested when at least 80% of them were ballon-like. The cells were washed in phosphate buffered saline (PBS), spotted on immunofluorescence slides (Biomerieux, Marcy L'Etoile, France), fixed with cold acetone:methanol (1:1) for 10 min and stored at −20°C. At the time of analysis, the slides were thawed and washed in distilled water. Sera were diluted in PBS starting from 1:20:10 μl was distributed on each well and incubated for 30 min at 37°C in a moist chamber for IgG detection. After rinsing, fluorescein isothiocyanate-labelled goat antihuman IgG antibody (Sigma, St. Louis, MO) was added to each well, and incubated for another 30 min at 37°C. The slides were rinsed again and examined with a fluorescence microscope (Leitz, Germany, 40x). Positive control sera at known titre and negative sera were used in each slide. Our IFA method was initially compared with a commercial assay (Stellar, Bio Systems, Inc., Columbia) and its sensitivity and specificity were the same. To avoid false positive results, positive samples were also tested for autoantibodies using Ramos cells (ATCC CRL 1596) and immunofluorescence assay, following the protocol described above [Kedes et al., 1996].

RESULTS

PCR and Genetic Variants

Sixteen of the 23 (69.6%) autologous BMT recipients were negative for HHV-6 DNA in PBMCs. The other patients (30.4%) had intermittent (five patients, 21.7%) or persistent (two patients, 8.7%) positivity for HHV-6 DNA, as illustrated in Table I. There was no difference between these two patients and the other five PCR positive patients as far as the engraftment and immunosuppressive regimen were concerned. None of the patients had clinical symptoms ascribed unequivocally to active HHV-6 infection. At the time of the transplant, two patients had HHV-6 DNA in PBMCs whereas the other patients developed positivity at different times. When the samples with HHV-6 DNA in PBMCs were tested for HHV-6 DNA in plasma, they were all negative. Four patients with positive PBMCs carried variant B, and the other three carried both A and B. None of the positive samples of a given patient showed any change in restriction pattern. Human herpesvirus type 6 DNA was not found in PBMCs of controls.

For statistical analysis, 25 autologous BMT procedures were considered, since two of the studied patients underwent transplant twice. The prevalence of HHV-6 DNA in transplant patients versus healthy subjects was not significantly different ($P = 0.08$, Fisher's exact test). The prevalence of HHV-6 DNA in PBMCs on day 30 versus day 180 was not statistically different ($P = 0.08$, Fisher's exact test).

Serology

All patients were without detectable HHV-6 antibodies except patients 15 and 18, who were positive from day 90 to 180 and from day 0 to 45, respectively. Nei-

TABLE I. HHV-6 DNA Detection By Nested PCR in PBMC and Respective Plasma Samples of Autologous BMT Recipients/Healthy Controls

BMT recipients	Day 0		Day 7		Day 14		Day 30		Day 45		Day 60		Day 90		Day 180	
	PBMC	pl	PBMC	pl	PBMC	pl	PBMC	pl	PBMC	pl	PBMC	pl	PBMC	pl	PBMC	pl
1	+ (A,B)[a]	–	–		+ (A,B)	–	–		+(A,B)	–	+(A,B)	–	–		–	
2	–		nd		–		–		–		–		–		–	
3	+ (A,B)	–	+ (A,B)	–	–		+ (A,B)	–	–		–		nd		–	
4	–		–		–		–		–		–		–		–	
5	–		–		+ (A,B)	–	–		+ (A,B)	–	–		–		+ (A,B)	–
6	–		nd		–		–		+ (B)		–		+ (B)	–	–	
7	–		+(B)[b]	–	–		–		–		–		+ (B)	–	–	
8a	–		–		–		–		–		–		–		–	
8b	–		–		–		–		–		–		–		–	
9	–		–		–		–		–		–		–		–	
10a	–		–		–		–		–		–		–		–	
10b	–		–		–		–		–		–		–		–	
11	–		–		–		–		–		+ (B)	–	+ (B)	–	+ (B)	–
12	–		–		–		–		–		–		–		–	
13	–		–		–		–		–		–		–		–	
14	–		–		–		–		–		–		–		–	
15	–		–		–		–		–		–		–		–	
16	–		–		–		–		–		–		–		–	
17	–		–		–		+ (B)	–	+ (B)	–	+ (B)	–	+ (B)	–	+ (B)	–
18	–		–		–		–		–		–		–		–	
19	–		–		–		–		–		–		–		–	
20	–		–		–		–		–		–		–		–	
21	–		–		–		–		–		–		–		–	
22	–		–		–		–		–		–		–		–	
23	–		–		–		–		–		–		–		–	
Healthy controls																
24	–												–		–	
25	–												–		–	
26	–												–		–	
27	–												–		–	
28	–												–		–	
29	–												–		–	
30	–												–		–	
31	–												–		–	
32	–												–		–	
33	–												–		–	

[a](A,B) = sample positive for both A and B HHV-6 DNA variants.
[b](B) = sample positive for B HHV-6 DNA variant.
Plasma HHV-6 Detection was performed only in samples whose PBMC were positive.

ther had HHV-6 DNA in PBMCs. The samples of patient 12 were positive for autoantibodies and were considered negative. All control samples were negative for HHV-6 antibodies.

DISCUSSION

Recent interest in the pathogenetic role of HHV-6 has revealed that although it is the etiological agent of exanthem subitum, clinical manifestations are usually due to reactivation in immunocompromised patients. The aim of the present study was to detect the reactivation of HHV-6 in autologous BMT recipients. The criteria selected to obtain a clear evaluation were based on HHV-6 DNA detection in PBMCs, but since DNA detection in PBMCs alone does not distinguish between latent and active infection [Carrigan, 1995], samples with HHV-6 DNA in PBMCs were analyzed subsequently for HHV-6 DNA in plasma. The presence of anti-HHV-6 antibodies was also evaluated.

A number of previous studies have described HHV-6 infection in patients undergoing organ transplantation [Osman et al., 1997; Singh et al., 1997]. Active infection has been demonstrated early [Singh and Carrigan, 1996] and late after allogeneic BMT, and seems to cause chronic myelosuppression [Knox and Carrigan, 1996; Rosenfeld et al, 1995]. Conversely, autologous BMT has been less investigated, since these patients are not immunosuppressed like allogeneic BMT recipients [Carrigan et al, 1991; Kadakia et al, 1996; Chan et al, 1997].

The results of the present study are in agreement with the study by Chan et al. [1997] which showed percentages of HHV-6 DNA positivity in PBMCs and plasma samples similar to ours. Conversely, the data obtained does not confirm the study of Kadakia et al. [1996] for several reasons: (1) very few of the patients monitored were seropositive, and one had autoantibodies; (2) only 30.4% of our autologous BMT recipients had intermittent or persistent positivity in PBMCs and none were positive for HHV-6 DNA in plasma specimens; (3) none of the patients developed clinical symptoms that could be attributed to HHV-6 infection/reactivation; and (4) 4/7 of the positives carried variant B and the other three carried both A and B variants.

None of the samples of a given patient changed restriction pattern during follow up. Since HHV-6 DNA in plasma is, in the authors' opinion, the most reliable marker of active viral infection, the intermittent or persistent finding of HHV-6 DNA in PBMCs was a clear sign of an increase in viral copy numbers, but was not sufficient to assess viral reactivation.

The present study showed an increasing number of HHV-6 DNA positive PBMC samples at longer times after transplant. The reason is not clear, but HHV-6 did not seem to be affected by immunosuppressive therapy, as it is in allogeneic BMT, for which immunosuppressive therapy is stronger and of longer duration. Furthermore, as there were only two HHV-6 DNA positive PBMC samples at time 0, it was not possible to ascribe a predictive value for HHV-6 infection to this finding unlike the case of allogeneic BMT [Yoshikawa et al., 1998]. PCR and serology data did not demonstrate any correlation. This finding is in keeping with the current opinion that HHV-6 serology is not a reliable marker of HHV-6 infection [Osman et al., 1997].

In conclusion, the present study did not demonstrate a clear reactivation of HHV-6 in autologous BMT recipients. However, long term study of the same population of patients could be performed to evaluate late active infection after the transplant and to ascribe a role as marker of reactivation to HHV-6 DNA positivity in PBMCs. This is suggested by evidence of increasing PBMCs positivity with time elapsing since transplant. The three patients with both genetic variants of HHV-6 could be monitored for a longer period to evaluate late graft failure and clinical signs, as demonstrated previously [Rosenfeld et al., 1995].

REFERENCES

Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim-van Dillen PME, van der Noordaa J. 1990. Rapid and simple method for purification of nucleic acids. J Clin Microbiol 28:495–503.

Braun DK, Dominguez G, Pellett PE. 1997. Human herpesvirus 6. Clin Microbiol Rev 10:521–567.

Carrigan DR. 1995. Human herpesvirus-6 and bone marrow transplantation. Blood 85:294–295.

Carrigan DR, Drobyski WR, Russler SK, Tapper MA, Knox KK, Ash RC. 1991. Interstitial pneumonitis associated with human herpesvirus-6 infection after marrow transplantation. Lancet 338:147–149.

Carrigan DR, Knox KK. 1994. Human herpesvirus 6 (HHV-6) isolation from bone marrow: HHV-6-associated bone marrow suppression in bone marrow transplant patients. Blood 84:3307–3310.

Chan PKS, Peiris JSM, Yuen KY, Liang RHS, Lau YL, Chen FE, Lo SKF, Cheung CY, Chan TK, Ng MH. 1997. Human herpesvirus-6 and human herpesvirus-7 infections in bone marrow transplant recipients. J Med Virol 53:295–305.

Cone RW, Hackman RC, Huang MW, Bowden RA, Meyers JD, Metcalf M, Zeh J, Ashley R, Corey L. 1993. Human herpesvirus 6 in lung tissue from patients with pneumonitis after bone marrow transplantation. N Engl J Med 329:156–161.

Drobyski WR, Dunne WM, Burd EM, Knox KK, Ash RC, Horowitz MM, Flomenberg N, Carrigan DR. 1993. Human herpesvirus-6 (HHV-6) infection in allogeneic bone marrow transplant recipients: evidence of a marrow-suppressive role for HHV-6 in vivo. J Inf Dis 167:735–739.

Drobyski WR, Knox KK, Majewski A, Carrigan DR. 1994. Fatal encephalitis due to variant B human herpesvirus-6 infection in bone marrow transplant recipients. N Engl J Med 330:1356–1360.

Kadakia MP, Rybka WB, Stewart JA, Patton JL, Stamey FR, Elsawy M, Pellett PE, Armstrong JA. 1996. Human herpesvirus 6: infection and disease following autologous and allogeneic bone marrow transplantation. Blood 87:5341–5354.

Kedes DH, Operskalski E, Busch M, Kohn R, Flood J, Ganem D. 1996. The seroepidemiology of human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus): distribution of infection in KS risk groups and evidence for sexual transmission. Nat Med 2:918–924.

Knox KK, Carrigan DR. 1996. Chronic myelosuppression associated with persistent bone marrow infection due to human herpesvirus-6 in a bone marrow transplant recipient. Clin Inf Dis 22:174–175.

Kwok S, Higuchi R. 1989. Avoiding false positives with PCR. Nature 339:237.

Luppi M, Marasca R, Barozzi P, Ferrari S, Ceccherini-Nelli L, Merelli E, Torelli G. 1993. Three cases of human herpesvirus-6 latent infection: Integration of viral genome in peripheral blood mononuclear cell DNA. J Med Virol 40:44–52.

Miller SA, Dykes DD, Polesky HF. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nuc Acid Res 16:1215.

Moschettini D, Balestri P, Fois A, Valensin PE. 1996. Acute encephalitis due to human herpesvirus 6. Clin Inf Dis 23:397–398.

Moschettini D, De Milito A, Catucci M, Marconi A, Rinina C, Bianchi Bandinelli ML, Valensin PE. 1998. Detection of human herpesvirus 6 and 7 in heart transplant recipients by a multiplex polymerase chain reaction method. Eur J Clin Microbiol Inf Dis 17:117–119.

Osman HK, Peiris JSM, Taylor CE, Karlberg JPE, Madeley CR. 1997. Correlation between the detection of viral DNA by the polymerase chain reaction in peripheral blood leucocytes and serological responses to human herpesvirus 6, human herpesvirus 7, and cytomegalovirus in renal allograft recipients. J Med Virol 53:288–294.

Rieux C, Gautheret-Dejean A, Challine-Lehmann D, Kirch C, Agut H, Vernant JP. 1998. Human herpesvirus-6 meningoencephalitis in recipient of an unrelated allogeneic bone marrow transplantation. Transplantation 65:1408–1411.

Rosenfeld CS, Rybka WB, Weinbaum D, Carrigan DR, Knox KK, Andrews DF, Shadduck RK. 1995. Late graft failure due to dual bone marrow infection with variants A and B of human herpesvirus-6. Exp Hematol 23:626–629.

Salahuddin SZ, Ablashi DV, Markham PD, Josephs SF, Sturzenegger S, Kaplan M, Halligan G, Biberfeld P, Wong-Staal F, Karamarky B, Gallo RC. 1986. Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. Science 234:596–601.

Secchiero P, Carrigan DR, Asano Y, Benedetti L, Crowley RW, Komaroff AL, Gallo RC, Lusso P. 1995. Detection of human herpesvirus 6 in plasma of children with primary infection and immunosuppressed patients by polymerase chain reaction. J Inf Dis 171: 273–280.

Singh N, Carrigan DR. 1996. Human herpesvirus-6 in transplantation: an emerging pathogen. Ann Int Med 124:1065–1071.

Singh N., Carrigan DR, Gayowski T, Marino IR. 1997. Human herpesvirus 6 infection in liver transplant recipients. Transplantation 64:674–678.

Wilborn F, Brinkmann V, Schmidt CA, Neipel F, Gelderblom,H, Siegert W. 1994. Herpesvirus type 6 in patients undergoing bone marrow transplantation: serologic features and detection by polymerase chain reaction. Blood 83:3052–3058.

Yamanishi K, Okuno T, Shiraki K, Takahashi M, Kondo T, Asano Y, Kurata T. 1988. Identification of human herpesvirus-6 as a causal agent for exanthem subitum. Lancet 1:1065–1067.

Yoshikawa T, Suzuki K, Ihira M, Furukawa H, Suga S, Asano Y, Kojima S, Kato K, Matsuyama T. 1998. Prediction of human herpesvirus 6 infection after allogeneic bone marrow transplantation. Blood 92:2597–2599.